From: Holleran, Anne
Sent: Tuesday, September 04, 2001 6:03 PM
To: STIC-ILL
Subject: refs. for 09/251,133

Examiner: Anne Holleran
Art Unit: 1642; Rm 8E03
Phone: 308-8892
Date needed by: ASAP

Please send me copies of the following :

1. Chien, J. et al. Mol. and Cell. Endocrinology (2001) 181(1-2): 69-79.
2. Chien, J. et al. Int. J. of Cancer (2001) 91(1): 46-54
3. Chien, J. et al. Oncogene (1999) 18(22): 3376-3382
4. Wong, E.C.C. et al. Proc. Amer. Assoc. for Cancer Res. (1997) 38: 288
5. Rayford, W. et al. Prostate (1997) 30(3): 160-166
6. Xue-Zhang, Q. et al. Endocrine (1995) 3(6): 445-451
7. Shah, G.V. et al. Endocrinology (1994) 134(2): 596-602
8. Rayford, W. et al. J. of Urology (1994) 151(5 suppl): 490A
9. Rayford, W.et al. J. of Urology (1993) 149(4 suppl): 479A
10. Shah, G.V. et al. Prostate (N.Y.) (1992) 21(2): 87-97
11. Sagol, O. et al. Annals of Medical Sciences (1999) 8(1): 14-21
12. Sussenot, O. et al. Prostate (1998) 36(suppl. 8): 43-51
13. Hanna, F.W. et al. J. Endocrinol. (1997) 152(2): 275-281
14. Sim, S.J. et al. Annals of Clinical and Laboratory Science (1996) 26(6): 487-495
15. Watanabe, K. et al. Fukushim J. Medical Science (1995) 41(2): 141-152
16. Esik, O. et al. European J. Gynaecological Oncology (1994) 15(3): 211-216

1

BEST AVAILABLE COPY

Molecular and Cellular Endocrinology

Calcitonin is a prostate epithelium-derived growth stimulatory peptide

Jeremy Chien [a], Yuan Ren [a], Yong Qing Wang [a], William Bordelon [b],
Evelin Thompson [b], Rodney Davis [c], Walter Rayford [d], Girish Shah [a],*

[a] *Department of Pharmaceutical Sciences, Texas Tech University Health Sciences center, Amarillo, TX 79106, USA*
[b] *Amarillo VA Medical Center, Amarillo, TX 79106, USA*
[c] *Department of Urology, Tulane University Medical Center, New Orleans, LA 70112, USA*
[d] *Department of Urology, Louisiana State University Medical Center, New Orleans, LA 70112, USA*

Abstract

Locally secreted growth factors and neuropeptides may play an important role in sustaining the growth of hormone-independent prostate cancer. Our previous studies have shown that calcitonin-like immunoreactive peptide (CTI) is secreted by primary prostate cells in culture, and its secretion from malignant prostate cells is significantly higher than benign cells. Exogenously added calcitonin (CT) induces DNA synthesis in serum-starved prostate cancer LNCaP and PC-3M cells. Present studies extended these findings by cloning cDNAs for CT and CT receptor (CT-R) from prostate cancer cells and studying the expression of CT and CT-R mRNA in prostate cancer cell lines and primary prostate tumor specimens.

The results have shown that PC-3 cells expressed CT, and not CT-R, mRNA, whereas CT-R, but not CT, mRNA was expressed by LNCaP cells. Conditioned media from PC-3 cells induced DNA synthesis of LNCaP cells, and this mitogenic response was abolished by anti-CT serum. Highly aggressive PC-3M cells co-expressed CT and CT-R mRNAs. CT also induced a twofold increase in DNA synthesis of primary prostate cells and anti-CT serum caused a 56% decline. In-situ hybridization histochemistry of archival prostate specimens has selectively localized CT and CT-R mRNA in basal epithelium of benign and low grade PC specimens, and these mRNAs were not detected in either luminal epithelium or stroma. In contrast, CT and CT-R mRNA were detected throughout the luminal epithelium of moderate and high-grade PC specimens. Most epithelial cells of low and moderately differentiated tumors expressed either CT or CT-R mRNA, suggesting that CT may serve as a paracrine factor. In contrast, CT and CT-R mRNAs were co-expressed by most tumor cells in advanced PC specimens. The cells expressing CT-R mRNA in primary tumors also co-expressed PCNA. These results, when combined with mitogenic actions of CT on primary prostate cells as well as PC cell lines, strongly support the role for CT in sustaining the growth of cancer cells. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Calcitonin; Prostate; Cancer; Basal; Growth

1. Introduction

Prostate carcinoma (PC) is the leading malignancy, in terms of incidence, and the second leading cause of cancer deaths in men (Chen et al., 1996). While the majority of PCs remain dormant for a long period of time, significant minorities of them display rapid growth and invasive characteristics (Newling et al., 1992, 1996). The mechanisms responsible for the latent growth of tumors in majority of cases and for rapid progression in minority cases have not been identified.

There is evidence to suggest that the progression of a primary tumor to an androgen-independent stage is associated with unregulated expression of paracrine/autocrine growth factors that may sustain the growth of tumor cells in the absence of androgens (Kadmon et al., 1991; Cohen et al., 1994; Huang et al., 1994). Indeed, locally synthesized peptides such as bombesin and neurotensin have been shown to exhibit autocrine growth stimulating activity in invasive human cancers (Sehgal

---

* Corresponding author. Tel.: +1-806-356-4000x325; fax: +1-806-356-4034.
E-mail address: girish@cortex.ama.ttuhsc.edu (G. Shah).

et al., 1994; Jongsma et al., 2000). Previous findings from this laboratory have shown that calcitonin-like immunoreactive substance(s) (CTIs) are secreted by explant-derived primary prostate cells (Shah et al., 1992). The studies have further shown that the secretion of CTIs from tumor-derived cells was significantly higher than that from benign prostatic hypertrophy (BPH)-derived cells. In addition, calcitonin (CT) stimulated the growth of human prostate cancer LNCaP and PC-3M cells (Shah et al., 1994; Chien et al., 2001). Since CT stimulated cyclic 3', 5'-adenosine monophosphate (cAMP) accumulation and also induced Gq-mediated signaling, it is likely that CT-induced increase in DNA synthesis of human prostate cancer cells may be mediated by either or both of these mechanisms (Shah et al., 1994).

There is evidence to suggest that the expression of CT gene may be induced in PCs, and prostate CT receptor (CT-R) has been localized to the prostate epithelium (Davis et al., 1989; Shah et al., 1992; Sim et al., 1996; Wu et al., 1996; Deftos et al., 1998; Iczkowski et al., 1998). However, there has been no systematic study investigating the expression of CT and its receptors in established PC cell lines and primary tumors, or examining its potential role as an important locally secreted growth factor. In the present study, we cloned CT and CT-R cDNAs from PC cell lines and used these as probes to investigate the expression of these mRNAs in established PC cell lines and primary tumors. The studies also developed a secretor/target co-culture model to examine the role of CT as a paracrine/autocrine growth factor. To extend the findings of CT actions in cell lines with PC pathophysiology, the studies also examined CT and CT-R mRNA expression in primary prostate tumors, and tested the role of CT as a mitogen by testing (1) whether immunoneutralization of endogenous CT reduces the growth of primary tumor cells and (2) whether cell populations expressing CT-R in primary prostate tumors are actively proliferating.

2. Materials and methods

2.1. Chemicals and reagents

Media and sera for tissue culture were obtained from GIBCO-BRL (Grand Island, NY). ^{3}H-Methyl thymidine was purchased from Radiochemical Center (Amersham, UK). All chemicals, unless otherwise stated, were obtained from Sigma Chemical Company (St. Louis, MO). Anti-CT serum used for immunoneutralization has been raised and characterized in this laboratory and has been successfully used to measure CTI levels in the conditioned prostate cell-culture media as well as for immunoneutralization and passive

immunization studies in tissue culture and in-vivo models (Shah et al., 1989, 1993, 1996).

2.2. PC cell lines and cell culture

LNCaP and PC-3 cell lines (CRL-1740 and CRL-7934, respectively) were purchased from ATCC (Manassas, VA). The PC-3M cell line was provided by Dr. Isiah Fidler (Anderson Cancer Center, Houston, TX). The cells lines were propagated and maintained in the complete medium (RPMI 1640 supplemented with L-glutamine, 5% fetal calf serum, 10 mM HEPES, 1.2% horse serum, 50 units/ml penicillin, 50 µg/ml streptomycin).

2.3. Patients and tissue specimens

The surgical pathology and autopsy specimen files at the Amarillo VA Medical Center were searched for all prostate needle biopsies, transurethral resection, prostatectomy and autopsy specimens with adenocarcinoma (particularly those with metastases), reported from 7/92 through 7/99. Some specimens used for in-situ hybridization were received from the Department of Urology at the Louisiana State University Medical Center. Twenty cases of pretreatment prostatic adenocarcinoma were selected, and these included 15 prostatectomy specimens. Among these, seven were TNM stage T2, eight were stage T3, three transurethral resections, one biopsy and one autopsy. Their Gleason scores varied from 3 to 10.

The protocol for the use of prostate tissue specimen has been approved by the institutional review boards at Amarillo and New Orleans.

2.4. RT-PCR: generation of partial CT and CT-R cDNAs probes

Total RNA from PC-3M and LNCaP prostate cancer cell lines (in logarithmic growth phase) was prepared by the method of Xie and Rothblum (Xie et al., 1991). In brief, the cells from a 100 mm culture dish were lysed in a single-step acid–guanidium thiocyanate–phenol extraction mixture. The nucleic acid fraction was separated by chloroform extraction and centrifugation. The total RNAs were precipitated in isopropanolol, treated with proteinase K, re-extracted in phenol: chloroform (1:1) and stored at −20 °C until used for reverse transcription.

One microgram of total RNA was used for reverse transcription (RT). Oligo dT primer annealing and reverse transcription was performed using SuperscriptII reverse transcriptase according to the manufacturer's protocol (GIBCO-BRL, Gaithersburg, MD). The following primer pairs (derived from previously published CT and CT-R mRNA sequences) were used for the

PCR reactions. Specifically, primer pairs for CT mRNA spanned exons 1 and 4 to ensure that we do not amplify genomic sequences. Since the gene structure of CT-R has not been described, we ensured that the RNA used for the RT-PCR was DNA-free, and the size of the amplified product matched with the length of the sequence spanning the amplimer pairs. The sequences of the primer pairs were as follows:

CT-forward: 5'-agagtcaccgcttcgcaa-3'; CT-reverse: 5'-ccagagaggaactacatgcatc-3'

CTR-forward: 5'-gtattgtcctatcattctgcc-3'; CTR-reverse: 5'-gccagcagttgtcattgaag-3'.

The PCR reaction was hot started by the addition of Taq polymerase after heating the reaction mix at 94 °C for 3 min. The amplification was performed for 35 cycles as follows: 94 °C for 20 s (denaturation); 58 °C for 20 s (annealing) and 72 °C for 90 s (extension). The amplified products from respective reactions were separated on 1% agarose gel, the bands were cut, and DNA samples were extracted and subcloned in pGEM-T vector (Promega Laboratories, Milwaukee, WI). The recombinant plasmids were amplified and sent for DNA sequencing at the Texas Tech University Biotechnology Facility.

The sequence of CT mRNA displayed 95% homology with the published CT mRNA sequence (emb/X97991). The mRNA sequence also contained exon 4 of the CT gene, which is specific for CT mRNA, and not for CGRP mRNA(gb/M31027). Likewise, the CT-R mRNA sequence expressed 95% homology with the published CT-R mRNA sequence derived from breast carcinoma isolate (dbj/AB022177). These cDNAs were then used as probes for the detection and/or quantitation of respective mRNAs in subsequent studies.

2.5. S1-nuclease protection assay

^{32}P-labeled sense and antisense riboprobes were synthesized using the 'Riboprobe In Vitro Transcription System' from Promega, and ^{32}P-UTP. SP6 and T7 RNA polymerases were used to transcribe antisense and sense probes, respectively, and then the reaction mix was treated with RNAse-free DNAse (Promega) to remove the DNA template. The probes were precipitated in alcohol, and the pellets were vacuum-dried, resuspended in DEPC-treated water, and stored at −80 °C until use.

Total RNA samples from prostate cancer LNCaP, PC-3 and PC-3M cell lines were extracted from the cultures in exponential phase. Twenty micrograms of total RNA were incubated with anti-sense CT and/or CT-R probes (approximately 500,000 cpm) for 18 h at 42 °C. Subsequently, the samples were treated with 50 U of S1 nuclease (Ambion, TX) for 30 min at 37 °C. The protected RNAs were precipitated and fractionated on 8 M urea–5% polyacrylamide gel. The gel was dried and autoradiographed, and the autoradiograms were scanned for the image analysis (Bio-Rad).

2.6. Tissue explant culture and ^{3}H-thymidine incorporation

Needle biopsy tissue and transurethrally resected prostate chips were collected aseptically and cultured as described before (Cockett et al., 1993). In brief, the tissues were cut to remove the burned areas and minced into $1 \times 2 \times 1$ mm sections. Approximately eight such sections were evenly distributed in a collagen-coated 25 cm^2 tissue-culture flask (Falcon, Oxnard, CA). The explants were fed with 1.0 ml of culture medium (RPMI 1640 supplemented with L-glutamine, 5% fetal calf serum, 12% horse serum, 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 10 ng/ml of 5α-androstan-17βol-3-one) and allowed to plate for 4 days at 37 °C in 95% air–5% CO$_2$. The medium was then changed to a low serum culture medium (Basic RPMI-1640 supplemented with 3% fetal bovine serum; other additions of the medium remained as before), and the cultures were maintained in low serum medium, which was changed every 3–4 days. It has been shown that primary cells cultured under these conditions are primarily of epithelial origin (Shah et al., 1992).

Cultured primary prostate cells in log phase were seeded at a density of 3×10^4 cells/well in 1 ml of RPMI-1640 growth medium in polylysine-coated 24-well culture plates. The growth rate was slowed down by overnight incubation in low serum containing medium (1% FCS only with no horse serum) followed by 2 h incubation in serum free basal medium. The cells were then treated with fresh basal medium containing either non-immune rabbit serum or rabbit anti-CT serum (final dilution 1:50). The incubations were continued for 24 h. Four hours prior to the termination of the assay, the cells received ^{3}H-thymidine (0.5 μCi/well). At the end of incubation, the cells were washed twice with PBS containing 100 μM unlabeled thymidine, and solubilized in Triton X-100 (0.1% vol/vol in distilled water). The incorporated ^{3}H-thymidine was quantified by liquid scintillation counting.

The results were expressed as dpm of ^{3}H-thymidine incorporated per 3×10^4 cells $\pm$ S.E.M. The data were analyzed by one-way ANOVA and the level of significance was derived from Newman–Keul's test.

2.7. Co-cultures of PC-3 and LNCaP cells

To further examine the role of endogenous CT in PC cell proliferation, a co-culture system was developed. PC-3 cells, which express CT mRNA and CT-I, were cultured separately in a Transwell™ upper chamber insert with the porous culture surface (24 mm diameter, 0.4 μm pore size, Costar, Cambridge, MA). LNCaP

cells (target cells) were cultured in the lower chamber. During the experimental period of 24 h, the upper chamber was inserted on top of the lower chamber so that LNCaP cells did not come into direct contact with PC-3 cells, but are exposed to PC-3 secretions. The experiment was divided into four groups: (A) untreated control; (B) medium + 10 μl of non-immune serum (NIS); (C) medium + 5 μl As-CT serum + 5 μl of NIS; and (D) received 10 μl of Anti-CT serum + medium. The incubations were continued for 24 h. Four hours prior to the termination of the assay, the cells received ^{3}H-thymidine (0.5 μCi/well). At the end of incubation, the cells were washed twice with PBS containing 100 μM unlabeled thymidine and solubilized in Triton X-100 (0.1% vol/vol in distilled water). The incorporated ^{3}H-thymidine in LNCaP cells was quantified by liquid scintillation counting. The data were analyzed as described above.

2.8. In-situ hybridization histochemistry for CT and CT-receptor (R) mRNA

2.8.1. Generation of riboprobes

Biotin-11-UTP labeled sense and antisense riboprobes were synthesized as described above. The riboprobes were precipitated in alcohol, and the pellets were vacuum-dried and resuspended in DEPC-treated water, and stored at −80 °C until use.

2.8.2. In-situ hybridization (ISH)

Five-micrometer-thick paraffin sections were first baked at 80 °C for 12 min and rinsed in xylene. The slides were then gradually rehydrated with successive passages through the graded alcohol solutions (100% alcohol, 95% alcohol, 75%, 50%, 20% alcohol and PBS), and incubated with 1 μM Proteinase K at 37 °C for 30 min. The slides were then washed in PBS (3 min × 2), incubated in 0.2 N HCl for 10 min at room temperature, and acetylated in acetic anhydride (0.5 ml/200 ml of 18.56 g/l of triethanolamine). To denature the secondary RNA structures, the sections were incubated in 2 × SSC at 70 °C for 15 min and quickly chilled at 4 °C. The sections were then incubated in the prehybridization buffer (4 × SSC, 50% deionized formamide) for 30 min at room temperature, and hybridized with either sense or anti-sense probes for CT or CT-R mRNA (in 40% deionized formamide, 10% dextran sulfate, 1% Denhardt's, 4 × SSC, 10 mM DTT, 1 mg/ml of yeast RNA, 1 mg/ml of salmon sperm DNA) at 42 °C overnight. Next day, the slides were washed in PBS (3 min × 2), and incubated with 100 pg/ml of RNase A for 30 min at 42 °C. Slides were then washed consecutively in 2 × SSC, 1 × SSC, and 0.5 × SSC for 5 min each, all at 42 °C. Slides were washed again with Tris buffer (0.15 M NaCl, 0.1 M Tris, pH 7.5) and incubated in the blocking solution

(1% sheep serum and 0.03% triton × 100 in Tris buffer) for 20 min at room temperature. This was followed by incubation with streptavidin–horse-radish peroxidase conjugate (1:200) for 3 h at room temperature. The slides were thoroughly washed with Tris buffers (pH 7.5 and 9.5) and, the color was developed using di-aminobenzidine-peroxide substrate (Zymed Laboratories, San Francisco, CA). The slides were washed in distilled water, counter-stained with hematoxylin, washed again, dehydrated in graded alcohol, and cover-slipped. The sections were then examined under Nikon Optiphot™ microscope, and digital images were captured by a Spot™ digital camera attached to the microscope.

2.9. Interpretation and analysis of in-situ hybridization results

Digital images from individual samples were analyzed as follows: cytoplasmic staining of prostate epithelium was scored on a scale from 0 (absent) to 4 + (strong and abundant). At least 50 cells per each field and 10 randomly selected fields per image were assessed. The staining was scored as the sum of the following two components.

1. The number of positive cells per field was scored as follows: < 5% as 0, 5c25% as 0.5, 35–50% as 1.0, 50–75% as 1.5 and 75–100% as 2.0.
2. The intensity of staining was scored as: 0.5 for trace, 1.0 for low, 1.5 for moderate, and 2.0 for strong.

Diagnosed Gleason grades were reassessed and compared with the staining intensity in a paired correlation for $n = 20$ cases. Among $n = 8$ cases with both PIN and PC, staining of these lesions was compared over the range of Gleason scores. Also evaluated were staining of lymphocytes, urothelium, seminal vesicles and stroma.

2.10. Combined CT-R ISH-PCNA immunohistochemistry

Anti-sense CT-R mRNA riboprobe was transcribed using digoxigenin 11-UTP (Boehringer Mannheim, Indianapolis, IN) as described in Section 2.8.2.

The PC sections were processed for CT-R mRNA ISH as described above. After washings and RNase A digestions, the sections were incubated with Tris-buffered saline (TBS) containing 0.1% Triton X-100, sheep anti-DIG-AP (Fab fragment) (1:200) and mouse anti-PCNA (Zymed Laboratories, 1:20) at room temperature for 3 h. After thorough washings with TBS, the samples were incubated with biotinylated anti-mouse IgG (1:200) at room temperature for 2 h and with ABC compound (1:100; Vector Technologies) for 1 h. After color development with diaminobenzidine (2 min), the sections were washed with TBS and incubated

with AP substrate containing NBT, BCIP and levamisole for 40 min at room temperature in the dark. The sections were then washed with Tris–EDTA buffer, distilled water, and dried in the air overnight. The sections were then mounted with permount and observed under Nikon Optiphot microscope. The images were captured by a Spot digital camera attached to the microscope.

3. Results

3.1. Expression of CT and CT-R mRNAs in PC cell lines

The results presented in Fig. 1 show that LNCaP cells expressed only CT-R mRNA but not CT mRNA, whereas PC-3 cells expressed only CT mRNA, and not CT-R mRNA. However, highly aggressive PC-3M cells expressed, CT and CT-R, mRNAs.

PC-3 cells stimulate ^{3}H-thymidine incorporation of LNCaP cells; anti-CT serum abolishes this activity:

Since PC-3 cells express CT mRNA, whereas LNCaP cells express CT-R and respond to CT, we developed a PC-3-LNCaP co-culture to test whether PC-3-derived CT is an endogenous mitogen for prostate cancer cells. The results presented in Fig. 2 show that the exposure
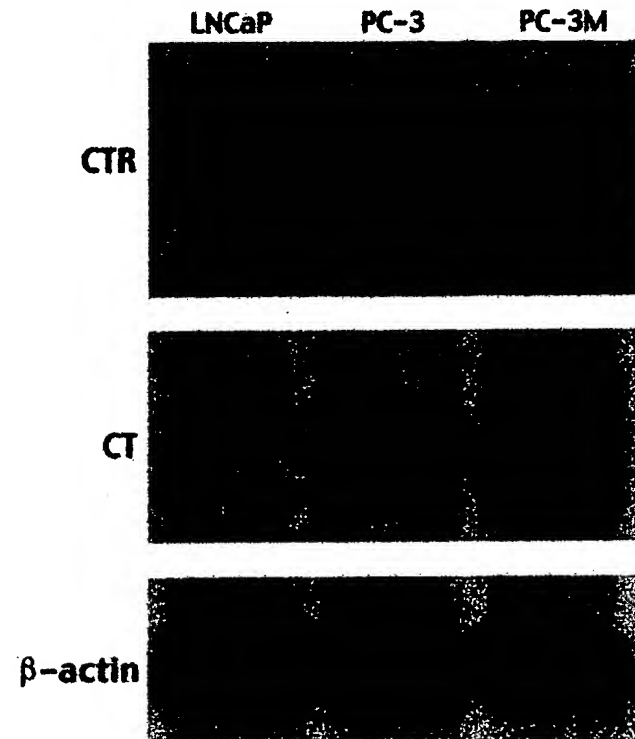

Fig. 1. Autoradiogram (24 h exposure) depicting the presence of CT, CT-R and β-actin mRNAs in LNCaP, PC-3 and PC-3M prostate cancer cell lines. The mRNA levels were analyzed by S1-nuclease protection assay as described in Section 2.
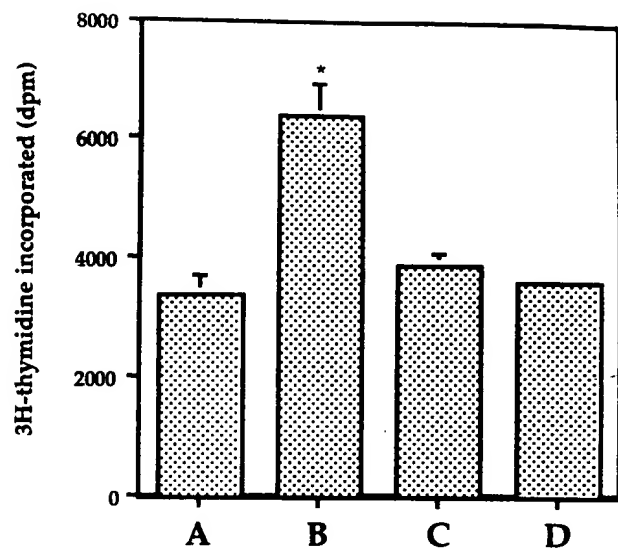

Fig. 2. Co-culture of PC-3 and LNCaP cells: effect on ^{3}H-thymidine incorporation of LNCaP cells. PC-3 cells were cultured in the upper insert (15,000 cells per insert), whereas LNCaP cells (15,000 cells/well) were cultured in 24-well culture plates. After the attachment period of 48 h, the cells were washed in a serum-free basal medium. The co-culture was set up by inserting PC-3 cell insert on top of LNCaP cell wells so that the LNCaP cells were exposed to PC-3 secretions without coming into direct contact with them. The co-culture was continued for 24 h, and 0.1 μCi of ^{3}H-thymidine was added during last 4 h. The LNCaP cells were then washed and solubilized, and the levels of incorporated ^{3}H-thymidine was determined as described in Section 2. Each experiment was done in triplicates, and the experiment was repeated three separate times. The results are expressed as mean dpm of ^{3}H-thymidine incorporated $\pm$ S.E.M. ($n = 9$) against the treatment. (A) LNCaP-LNCaP co-culture with non-immune serum (NIS) (1:50) in the lower chamber; (B) PC-3-LNCaP co-culture with NIS (1:50) in the lower chamber; (C) PC-3-LNCaP co-culture with rabbit anti-CT serum (As-CT) (1:100) in the lower chamber; (D) PC-3-LNCaP co-culture with As-CT (1:50) in the lower chamber. *$P < 0.05$ (homologous vs. heterologous co-cultures) One-way ANOVA and Newman–Keuls test.

of PC-3 secretions caused over a twofold increase in LNCaP cell thymidine incorporation (B), and the presence of anti-CT serum abolished this increase (C and D). The antiserum has been well characterized and has been used to immunoneutralize endogenous CT in cultured pituitary cells (Shah et al., 1993, 1996).

3.2. Effect of CT on ^{3}H-thymidine incorporation of primary prostate cells

Previous studies have shown that CTI is secreted by primary prostate cells, and its secretion is higher in malignant cells (Shah et al., 1992). CT also induces a significant increase in DNA synthesis of LNCaP and PC-3M cells (Shah et al., 1994; Chien et al., 2001). In the present study, we tested (1) whether CT induces mitogenic response in primary prostate cells and (2) whether immunoneutralization of endogenously secreted CTI by anti-CT serum reduces their rate of

^{3}H-thymidine incorporation. PC-derived primary cells were treated with either 10 nM CT or anti-CT serum (1:50) for 24 h. Concurrent controls received an equivalent amount of vehicle (10 μl basal medium for CT, or non-immune serum for anti-CT serum, 1:50). CT (10 nM) caused a significant, twofold, increase in ^{3}H-thymidine incorporation as compared to control, whereas anti-CT serum (As-CT) caused a significant, 56% decline in the rate ^{3}H-thymidine incorporation (Fig. 3).

3.3. Expression of CT and CT-R mRNA expression in primary prostate tumors: regional localization and variation with tumor grade

Consistent with our previous results on the presence of CT-immunopositive cells in the epithelium of PC (Iczkowski et al., 1998), present results have also localized CT and CT-R mRNAs in the epithelial, and not the stromal, compartment of the prostate (Fig. 4). CT and CT-R mRNAs were undetectable in most BPH specimens. Only in four out of 20 BPH cases did we detect the presence of CT mRNAs in four cases, while CT-R mRNAs were detected in two cases (Table 1).
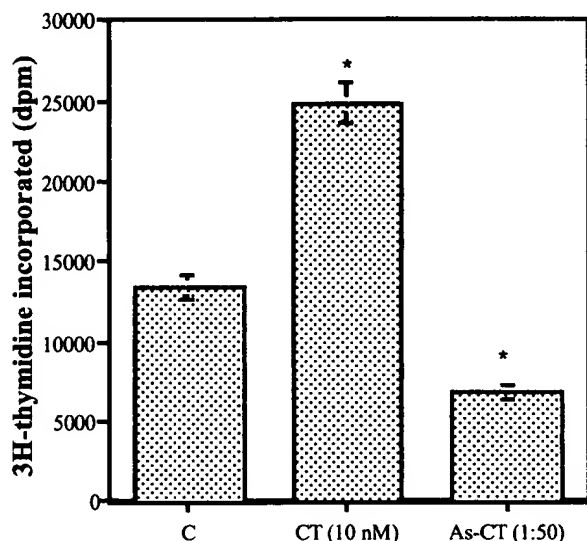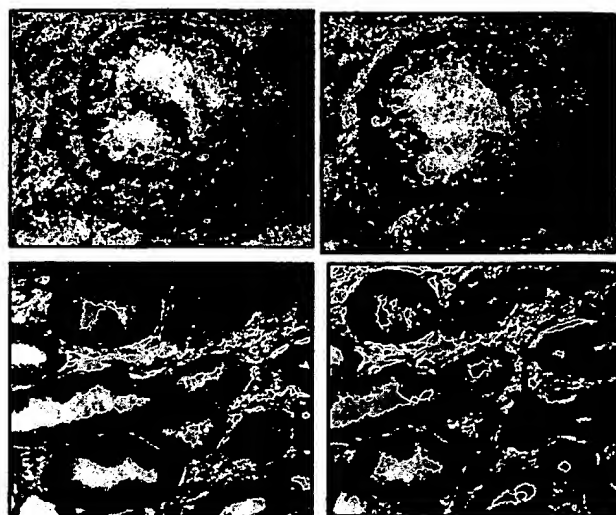

Fig. 4. (A, B) CT and CT-R mRNAs are selectively expressed by cells of basal epithelium of a well-differentiated tumor (Gleason sum of 4.0). (C) Expression of CT mRNA. (D) CT-R mRNA expression (100 × magnification) in a moderately differentiated tumor. Arrows indicate the location of positive cell populations.

Fig. 3. Effect of CT immunoneutralization on ^{3}H-thymidine incorporation of primary prostate cancer cells. Primary prostate cells were obtained from tissue explant cultures as described in Section 2 and plated in 24-well plates at a density of 30,000 cells per well. After the attachment in the complete medium, the cells were treated with 1:50 concentrations of either rabbit non-immune serum (NIS) or equivalent concentrations of rabbit anti-CT serum (As-CT), and incubated for 24 h in serum-free basal medium. The cells also received 0.1 μCi of ^{3}H-thymidine during last 4 h. At the end of incubation, the cells were washed and solubilized, and incorporated ^{3}H-thymidine was determined as described in Section 2. Each experiment was done in triplicates, and the experiment was repeated three separate times. The results are plotted as mean dpm thymidine incorporated ± S.E.M. ($n = 9$) against the treatment (NIS vs. As-CT). *$P < 0.05$ (NIS vs. As-CT) One-way ANOVA and Newman–Keuls test.

These mRNAs in BPH and low-grade tumors (up to Gleason score 4.0) were exclusively localized in the basal layer of the epithelium (Fig. 4A and B). In contrast, CT and CT-R mRNA species were distributed throughout the luminal epithelium of intermediate and high-grade PC specimens (Fig. 4C and D).

At least among the small number of tumor specimens evaluated in the present study, CT mRNA expression (as assessed by the intensity as well as the number of positive cells per field) increased progressively from low grade to intermediate grade (Fig. 5B and C, Table 1). However, the intensity of CT mRNA staining in high-grade tumors seemed less than that in intermediate grade tumors (Fig. 5D). Unlike CT mRNA, CT-R mRNA was almost undetectable in BPH or low-grade PC (up to Gleason sum of 4) specimens by the present technique (Fig. 5E). However, CT-R mRNA became detectable in the epithelia of moderately differentiated tumors, and its expression increased with the progression of tumor (Fig. 5F–H, Table 2). In aggressive tumors, CT and CT-R mRNAs were also expressed by tumor cells invading stroma (data not shown). However, a larger number of samples will have to be exam-

Table 1
CT mRNA expression in primary tumor specimens: staining score

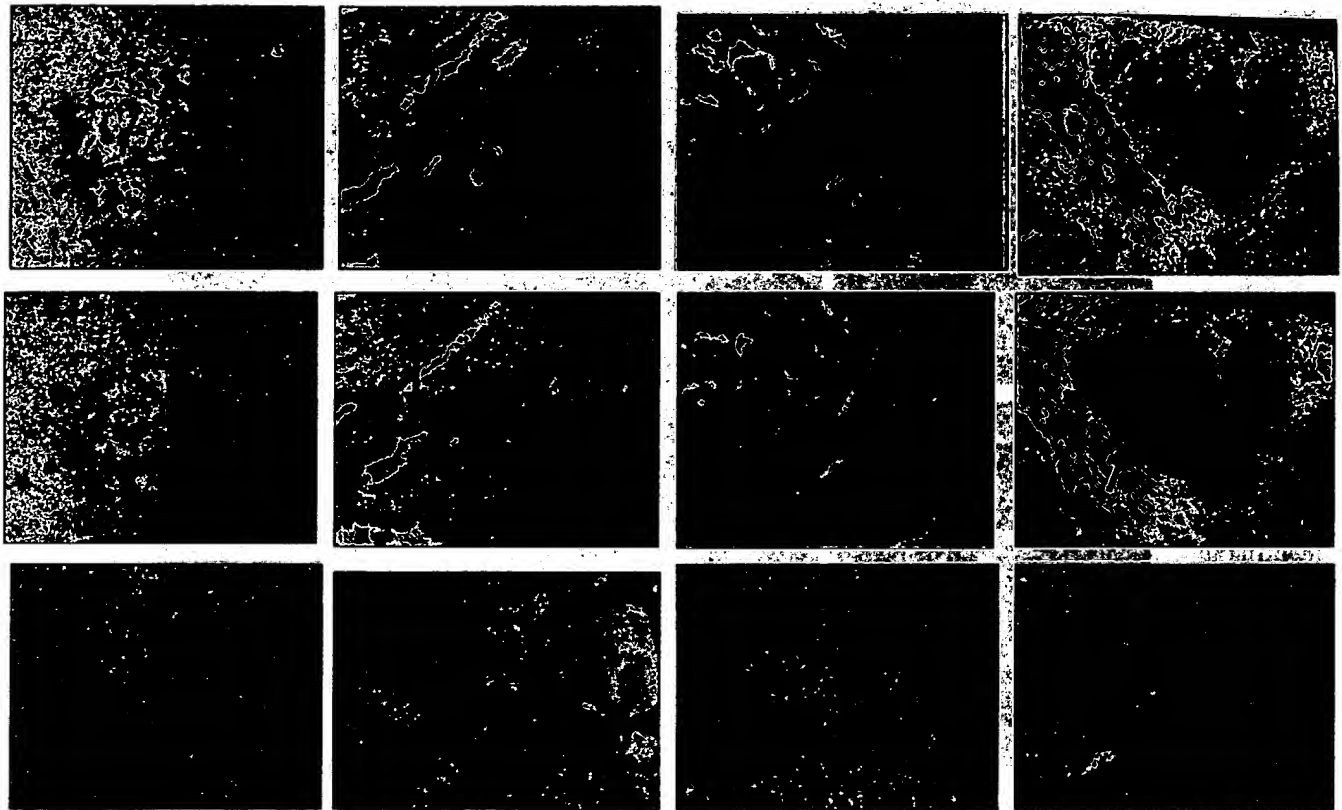| Grade	n	0	1+	2+	3+	4
BPH	20	16	4			
PIN	7		7			
Gleason Score 2–4	9		1	6	2	
Gleason Score 5–6	5				1	4
Gleason Score 7–10	6		1	3	1	1

Fig. 5. (A–D) in top row: Typical photomicrographs (100 × magnification) presenting the profiles of CT mRNA expression in BPH (A); well differentiated (upto Grade 4.0, B); moderately differentiated (upto Grade 7.0, C) and poorly differentiated tumors (> Grade 7.0, D). (E–H) in middle row: Representative photomicrographs of CT-R mRNA expression in various prostate specimens (100 × magnification). Representative examples of BPH (E), well differentiated (F); moderately differentiated (G), and poorly differentiated (H) tumors. (I–L) in bottom row: Representative sense controls (either CT or CT-R mRNA) for BPH (I), well differentiated (J), moderately differentiated (Panel K) and poorly differentiated tumors (L).

ined before any definitive relationship between CT and/ or CT-R mRNA expression and tumor grade can be established.

3.4. Expression of CT and CT-R mRNAs by primary tumor cells: moderately differentiated very poorly differentiated

Expression of CT and CT-R mRNA in primary tumor cells was further characterized using dual fluores-

Table 2
CT-R mRNA expression in primary tumor specimens: staining score

Grade	n	0	1+	2+	3+	4
BPH	20	18	2			
PIN	7	7				
Gleason Score 2–4	9	4	5			
Gleason Score 5–6	7	3			3	1
Gleason Score 7–10	4			1	2	1

Correlation co-efficient = 0.850 (n = 21; for Gleason sum between 2 and 7).

cent probes. The results presented in Fig. 6 demonstrate that cells in a moderately differentiated tumor expressed either CT mRNA (red, upper panels A and C), CT-R mRNA (green, upper panels B and C) or co-expressed both (yellow, upper panel C) in different regions. In contrast, most cells displayed the co-expression of both, CT (lower panels A and C) and CT-R mRNAs (lower panels B and C) in poorly differentiated tumors (> 7.0).

3.5. CT-R mRNA is co-localized in PCNA-positive cell populations of primary prostate tumors

Since previous studies have shown that CT induced a mitogenic response in PC cell lines (Shah et al., 1994; Chien et al., 2001), the present studies investigated whether CT affects the mitogenic activity of target cell populations (those expressing CT-R) in a primary tumor. This was indirectly examined by co-localization of CT-R mRNA with a proliferation marker PCNA. Fig. 7 is a representative photomicrograph of these results
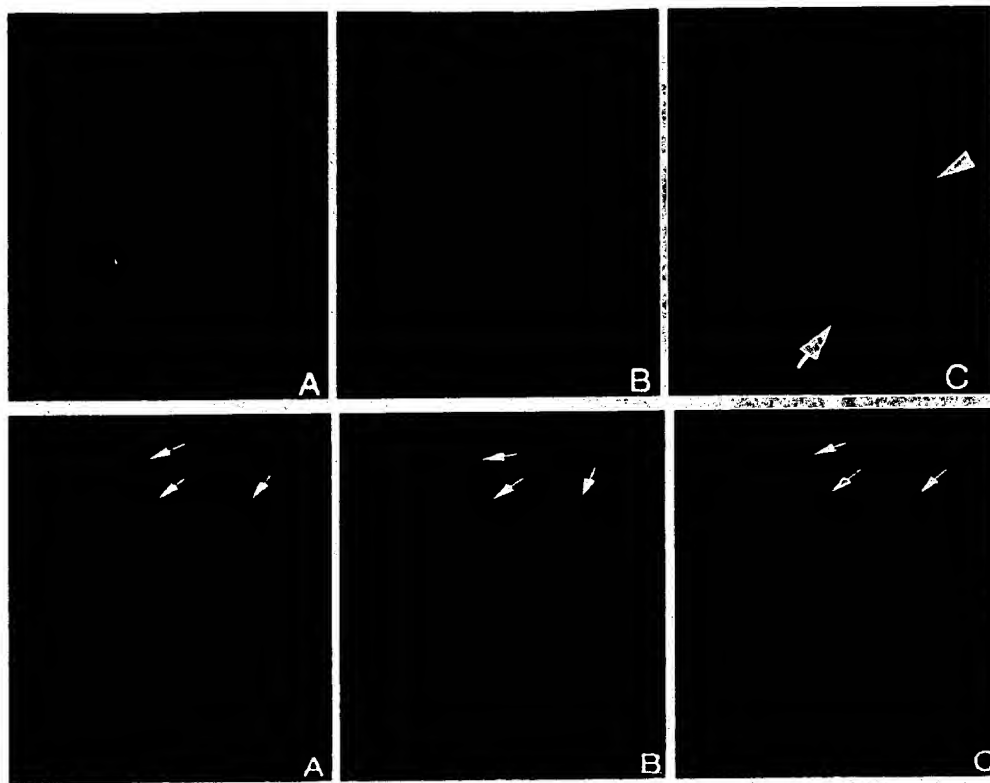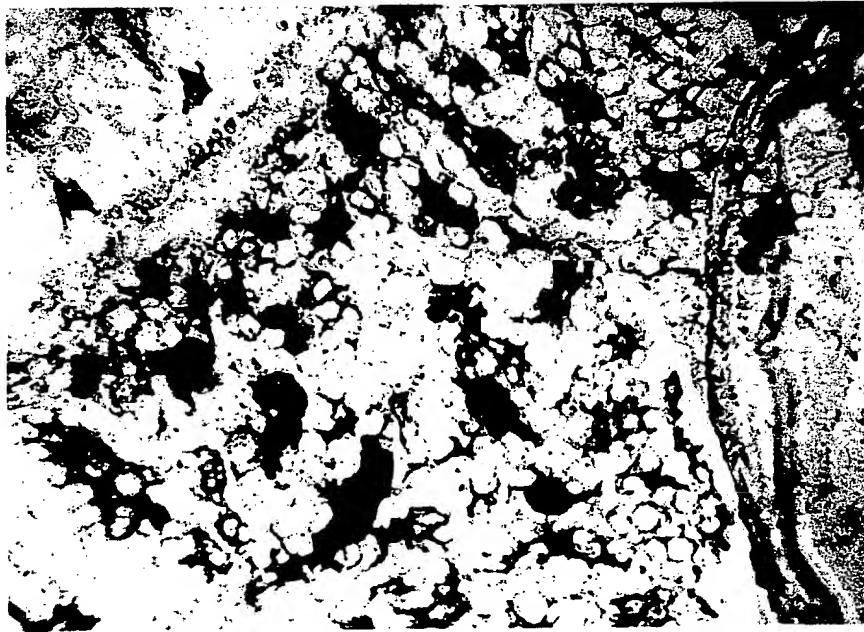
Fig. 6

Fig. 7

Fig. 6. Double fluorescence in-situ hybridization for CT- and CT-R mRNAs in the same section. Riboprobe for CT mRNA was labeled with TRITC-conjugated dCTP, whereas that for CT-R mRNA was labeled with FITC-conjugated UTP as described in Section 2. The upper left panel depicts a representative fluoromicrograph of CT mRNA expression (rhodamine, A) in moderately differentiated tumors (400 × magnification). The upper center panel (B) depicts CT-R mRNA expression in the same field (as in A). The upper (C) panel presents the expression of both mRNAs in the same field (as in A and B). The red florescence suggests the expression of CT mRNA alone (A and C); green fluorescence (B and C) is indicative of CT-R mRNA expression alone, whereas yellow fluorescence (C) is suggestive of a cell population co-expressing CT and CT-R mRNAs. Lower panels (A–C) present the profiles of CT and CT-R mRNAs in a poorly differentiated tumor section. Lower panel (A) depicts a representative fluoromicrograph of CT mRNA expression (rhodamine) in poorly differentiated tumors (400 × magnification). The B panel depicts CT-R mRNA expression in the same field (as in A). C presents the expression of both mRNAs in the same field (as in A and B).

Fig. 7. Combined CT-R mRNA in-situ hybridization-PCNA immunohistochemistry in a moderately differentiated tumor. Prostate tumor sections were probed for CT mRNA using a riboprobe labeled with UTP-digoxigenin, and this was followed with PCNA immunohistochemistry as described in Section 2. A representative photomicrograph (400 × magnification) reveals the co-expression of CT-R mRNA (blue cytoplasmic stain) and PCNA (brown nuclear stain) in the majority of CT-R mRNA-positive cell populations.

and demonstrates that most of CT-R mRNA-positive cells (indicated by blue cytoplasmic stain) in primary tumors co-express PCNA (brown nuclear stain). The number of cells expressing either PCNA alone, CT-R mRNA alone or those co-expressing CT-R mRNA and PCNA were counted. Of the total of 1000 cells counted, CT-R mRNA co-localized with 88% of total PCNA-positive cells.

4. Discussion

Previous findings from this and other laboratories have shown that CT induces DNA synthesis of LNCaP and PC-3M, but not PC-3 cells (Horoszewicz et al., 1983; Shah et al., 1994). To further characterize differential actions of CT on these prostate cancer cell lines, we examined the expression profiles of mRNA for CT and its receptor. The results demonstrate that andro-gen-refractory PC-3 cells express CT mRNA (Passaniti et al., 1992) and can serve as a model for CT-secreting prostate cells. Since they do not express CT-R mRNA, they do not respond to CT (Horoszewicz et al., 1983). LNCaP cells, which are indolent and androgen-respon-sive, express only CT-R, and not CT mRNA and respond to CT with increased cell proliferation (Horoszewicz et al., 1983; Shah et al., 1994). PC-3M cells, which are androgen-refractory and highly invasive (Kozlowski et al., 1984), co-express CT and CT-R mRNAs. With the expression of CT and its receptor, PC-3M cells become autonomous with respect to the 'CT system'. This possibility is supported by our previous findings that anti-CT serum causes a significant and dose-dependent decrease in proliferation of PC-3M cells (Chien et al., 2001).

Since PC-3 cells express CT and LNCaP cells express CT-R, we examined the effect of PC-3 secretions on LNCaP cell proliferation in a co-culture system. The results have shown that PC-3 secretions stimulated DNA synthesis of LNCaP cells, and this was reversed by anti-CT serum. Since the anti-CT serum is highly specific and effective in neutralizing endogenous CT (Shah et al., 1989, 1993, 1996), these results suggest that endogenously secreted CT could serve as an important paracrine/autocrine growth factor for prostate cancer cells. To further demonstrate the role of CT as an important mitogenic factor in primary PC pathophysi-ology, we examined the effect of CT and anti-CT serum on proliferation of primary prostate cultures. Since CT-I is secreted by primary cultures of prostate cancer cells (Shah et al., 1992), addition of anti-CT serum in the culture would immunoneutralize CT and thereby depletes its availability. Again, anti-CT serum caused a significant decline in DNA synthesis of primary PC cells. In contrast, exogenously added CT increased DNA synthesis of these cells. These results, when con-

sidered together with upregulation of CT expression in PC (Shah et al., 1992; Chien et al., 2001), further support our hypothesis that CT is an important paracrine/autocrine peptide that, along with other lo-cally secreted mitogens, plays an important role in sustaining proliferation of malignant cells. Having shown that prostate-derived CT can serve as a potent growth promoter for PC cells, we decided to extend these findings by investigating the expression of CT and its receptors in primary prostate tumors as well as its potential role.

The present studies with prostate specimens have shown that the mRNAs for CT and its receptor were selectively localized in prostate epithelium, and no mR-NAs for these genes were detected in the stroma. Fur-ther, mRNAs for CT and its receptor were selectively localized only in the basal layer of benign epithelium, and its complete absence in the luminal compartment was clearly evident. In contrast, these mRNAs were expressed by most cells in the luminal compartment of malignant prostates. The complete absence of these mRNA species in benign luminal epithelium, but their continued expression by malignant luminal cells, would suggest that the extinction of CT expression may be one of the several important events associated with normal differentiation process, and that malignant cells have not completed this process. Alternatively, malignant luminal cells may have dedifferentiated, and this pro-cess may have switched on the expression of CT, and possibly several other genes, in these cells (Liu et al., 1997). The significance of the expression of CT gene in basal epithelium, its extinction with differentiation, and its effect on the growth of prostate epithelium remain to be elucidated. These results raise several interesting possibilities such as (1) the differentiation of basal cells in malignant epithelium is arrested at a stage prior to the normal switching off of CT gene expression; (2) continued expression of CT, and possibly other neu-roendocrine factors in these cell populations, sustains their proliferation; (3) CT prevents the expression or actions of factors that support or sustain the process of terminal differentiation; (4) factors that upregulate CT gene expression are abundantly available in malignant epithelium; in contrast, these factors are downregulated as soon as basal cells differentiate into luminal cells.

Our previous studies have shown that CT induces mitogenic and invasive responses in LNCaP and PC-3M prostate cancer cells (Shah et al., 1994; Chien et al., 2001). To examine the possibility that CT may induce similar response in primary tumors, we tested whether the cells expressing CT-R mRNA are actively prolifer-ating. This was accomplished by co-localizing CT-R mRNA with PCNA. It has been shown that PCNA (36 kDa) is produced in G1 and S phases of the cell cycle (Tan et al., 1986). Because it is an auxiliary protein of DNA polymerase δ, PCNA is required for DNA repli-

cation (Tan et al., 1986). Several studies indicate that PCNA expression is a reliable paradigm for cellular proliferation in tissues from various species (Van Dierendock et al., 1991). Present results that a majority of CT-R mRNA-positive cells also expressed PCNA suggest that the cells expressing CT-R are actively proliferating and not terminally differentiated, cells.

Previous findings from this laboratory have shown that CT-I is secreted by primary prostate epithelial cells, and its secretion is upregulated in cancer cells (Shah et al., 1992; Iczkowski et al., 1998). The present results confirm and extend these findings by demonstrating that the expression pattern of CT and its receptors is dramatically altered with tumor progression. For example, the spatial distribution of CT and CT-R mRNA positive cells in low and intermediate grade tumors seemed to be distinct. In contrast, CT and CT-R mRNA were co-expressed by most tumor cells in high-grade tumors. These results raise the possibility that CT may be a paracrine factor during earlier stages of tumor but becomes an autocrine factor with tumor progression. With shift from a paracrine factor to an autocrine loop 'CT system' may provide the tumor cells a selective growth advantage. This, in turn, may provide them with selective growth advantage. This possibility is supported by the findings that all low-grade tumor cells and less invasive LNCaP and PC-3 cells express either CT or CT-R; and almost all cells in poorly differentiated tumors as well as PC-3M cells display aggressive growth characteristics, also co-express CT and CT-R mRNAs, and anti-CT serum inhibits their growth (Chien et al., 2001).

In conclusion, the present results have shown that CT is an important prostate-derived paracrine factor and plays a critical role in the growth of prostate epithelium. CT transforms from a paracrine into an autocrine factor in advanced PC and may play a role in invasive growth of PC cells. The growth-promoting actions of CT are corroborated in an in-vitro co-culture model using PC-3 and LNCaP PC cell lines, as well as in primary cultures.

Acknowledgements

References

Chen, C., Poulin, R., Labrie, F., 1996. Large Shionogi tumors lose their responsiveness to flutamide treatment. J. Steroid Biochem. Mol. Biol. 58, 489–494.

Chien, J., Shah, G.V., 2001. Role of stimulatory guanine nucleotide binding protein (Gsa) in proliferation of PC-3M prostate cancer cells. Int. J. Cancer 91, 46–54.

Cockett, A.T., di Sant'Agnese, P.A., Gopinath, P., Schoen, S.R., Abrahamsson, P.A., 1993. Relationship of neuroendocrine cells of prostate and serotonin to benign prostatic hyperplasia. Urology 42, 512–519.

Cohen, M.K., Arber, D.A., Coffield, K.S., Keegan, G.T., McClintock, J., Speights, V.O.J., 1994. Neuroendocrine differentiation in prostatic adenocarcinoma and its relationship to tumor progression. Cancer 74, 1899–1903.

Davis, N.S., DiSant'Agnese, P.A., Ewing, J.F., Mooney, R.A., 1989. The neuroendocrine prostate: characterization and quantitation of calcitonin in the human prostate gland. J. Urol. 142, 884–888.

Deftos, L.J., 1998. Granin-A, parathyroid hormone-related protein, and calcitonin gene products in neuroendocrine prostate cancer. Prostate. Suppl. 8, 23–31.

Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Chu, T.M., Mirand, E.A., Murphy, G.P., 1983. LNCaP model of human prostatic carcinoma. Cancer. Res. 43, 1809–1818.

Huang, Z.Y., Meng, F.Q., Chen, H., 1994. Relation between neuroendocrine expression of prostatic carcinoma and prognosis]. Chung. Hua. I. Hsueh. Tsa. Chih. 74, 23–25, 62.

Iczkowski, K.A., Pantazis, C.G., Long, J., Noble, M.J., Shah, G.V., 1998. Expression of calcitonin-like peptide is increased in prostatic adenocarcinoma. J. Uro. Pathol. 8, 135–147.

Jongsma, J., Oomen, M.H., Noordzij, M.A., Romijn, J.C., van der Kwast, T.H., Schroder, F.H., van Steenbrugge, G.J., 2000. Androgen-independent growth is induced by neuropeptides in human prostate cancer cell lines. Prostate Suppl. 42, 34–44.

Kadmon, D., Thompson, T.C., Lynch, G.R., Scardino, P.T., 1991. Elevated plasma chromogranin-A concentrations in prostatic carcinoma. J. Urol. 146, 358–361.

Kozlowski, J.M., Fidler, I.J., Campbell, D., Xu, Z.L., Kaighn, M.E., Hart, I.R., 1984. Metastatic behavior of human tumor cell lines grown in the nude mouse. Cancer Res. 44, 3522–3529.

Liu, A.Y., True, L.D., LaTray, L., Nelson, P.S., Ellis, W.J., Vessella, R.L., Lange, P.H., Hood, L., van den Engh, G., 1997. Cell–cell interaction in prostate gene regulation and cytodifferentiation. Proc. Natl. Acad. Sci. USA 94, 10705–10710.

Newling, D.W., 1992. What is 'progression' in prostate cancer? Prostate Suppl. 4, 139–143.

Newling, D.W., 1996. The management of hormone refractory prostate cancer. Eur. Urol. 29 (Suppl. 2), 69–74.

Passaniti, A., Isaacs, J.T., Haney, J.A., Adler, S.W., Cujdik, T.J., Long, P.V., Kleinman, H.K., 1992. Stimulation of human prostatic carcinoma tumor growth in athymic mice and control of migration in culture by extracellular matrix. Int. J. Cancer 51, 318–324.

Sehgal, I., Powers, S., Huntley, B., Powis, G., Pittelkow, M., Maihle, N.J., 1994. Neurotensin is an autocrine trophic factor stimulated by androgen withdrawal in human prostate cancer. Proc. Natl. Acad. Sci. USA 91, 4673–4677.

Shah, G.V., Pedchenko, V., Stanley, S., Li, Z., Samson, W.K., 1996. Calcitonin is a physiological inhibitor of prolactin secretion in ovariectomized female rats. Endocrinology 137, 1814–1822.

Shah, G.V., Kacsoh, B., Seshadri, R., Grosvenor, C.E., Crowley, W.R., 1989. Presence of calcitonin-like peptide in rat milk: possible physiological role in regulation of neonatal prolactin secretion. Endocrinology 125, 61–67.

Shah, G.V., Noble, M.J., Austenfeld, M., Weigel, J., Mebust, W.K., 1992. Presence of calcitonin-like immunoreactivity in human prostate gland: evidence for iCT secretion by cultured prostate cells. Prostate 21, 87–97.

Shah, G.V., Deftos, L.J., Crowley, W.R., 1993. Synthesis and release of calcitonin-like immunoreactivity by anterior pituitary cells: evidence for a role in paracrine regulation of prolactin secretion. Endocrinology 132, 1367–1372.

Shah, G.V., Rayford, W., Noble, M.J., Austenfeld, M., Weigel, J., Vamos, S., Mebust, W.K., 1994. Calcitonin stimulates growth of human prostate cells through receptor-mediated increase in cyclic adenosine 3′,5′-monophosphates and cytoplasmic Ca^{2+} transients. Endocrinology 134, 596–602.

Sim, S.J., Glassman, A.B., Ro, J.Y., Lee, J.J., Logothetis, C.J., Liu, F.J., 1996. Serum calcitonin in small cell carcinoma of the prostate. Ann. Clin. Lab. Sci. 26, 487–495.

Tan, C.K., Castillo, C., So, A.G., Downey, K.M., 1986. An auxillary protein for DNA polymerase delta from fetal calf thymus. J. Biol. Chem. 261, 1231–1236.

Van Dierendock, J.H., Wijsman, J.H., Kejizer, R., Cornelisse, C.J., 1991. Cell-cycle-related staining patterns of anti-proliferating cell nuclear antigen monoclonal antibodies. Comparison with BrdU labeling and Ki-67 staining. Am. J. Pathol. 138, 1165–1172.

Wu, G., Burzon, D.T., di Sant'Agnese, P.A., Schoen, S., Deftos, L.J., Gershagen, S., Cockett, A.T., 1996. Calcitonin receptor mRNA expression in the human prostate. Urology 47, 376–381.

Xie, W.Q., Rothblum, L.I., 1991. Rapid, small-scale RNA isolation from tissue culture cells. Biotechniques 11, 324, 326–324, 327.